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Expression of ACC oxidase genes differs among sex genotypes and sex phases in cucumber

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Abstract

Ethylene has been implicated as a sex-determining hormone in cucumber: its exogenous application increases femaleness, and gynoecious genotypes were reported to produce more ethylene. In this study, three full-length ACC oxidase cDNAs were isolated from cucumber floral buds. RFLP analysis of a population that segregates for the *F* (femaleness) locus indicated that *CS-ACO2* is linked to *F* at a distance of 8.7 cM. Expression of two of the genes, *CS-ACO2* and *CS-ACO3*, was monitored in flowers, shoot tips and leaves of different sex genotypes. *In situ* mRNA hybridization indicated different patterns of tissue- and stage-specific expression of *CS-ACO2* and *CS-ACO3* in developing flowers. *CS-ACO3* expression in mid-stage female flowers was localized to the nectaries, pistil and in the arrested staminoids, whereas *CS-ACO2* transcript levels accumulated later and were found in placental tissue, ovary and staminoids. In male flowers, petals and nectaries expressed both genes, whereas *ACO2* expression was strong in pollen of mature flowers. In young buds, strong expression was observed along developing vascular bundles. Four sex genotypes were compared for *CS-ACO2* and *CS-ACO3* expression in the shoot apex and young leaf. *FF* genotypes had higher transcript levels in leaves but lower levels in the shoot apex and in young buds, as compared to *ff* genotypes; the shoot-tip pattern is, therefore, inversely correlated with femaleness, and the possibility of a feedback inhibition mechanism underlying such correlation is discussed. The two *CS-ACO* genes studied displayed a differential response to ethrel treatment in different organs and sex genotypes, further demonstrating the complexity of the mechanisms controlling ethylene production during cucumber floral development.

Introduction

Flower development has been studied extensively in model plants that bear bisexual flowers, such as *Arabidopsis*, *Petunia* and *Antirrhinum* (Ma, 1994). Monoecious and dioecious plants bear, as a result of sex expression mechanisms, separate male and female flowers that develop from potentially bisexual floral buds (Dellaporta and Calderon-Urrea, 1993; Grant *et al.*, 1994). Such plants have, therefore, additional levels of control over flower development, as compared to the more common hermaphrodite plants, and these mechanisms are of great basic and applicative interest. *Cucumis sativus* (cucumber) is a monoecious species in which plant sex expression has been extensively studied (reviewed by Frankel and Galun, 1977; Perl-Treves, 1999). The cucumber embryonal flower bud has both stamen and ovary primordia (Atsmon and Galun, 1960), but later (in most varieties) a unisexual flower develops. Clusters of male flowers, or single female flowers, appear in the leaf axils. Cucumber sex expression typically follows a three-phase-pattern: a male phase with only staminate flowers, a mixed phase, and a continuous-female phase.

Genes that affect sex expression have been identified by geneticists and breeders, and can be combined to produce sex types other than monoecious. Such

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AF033581 (*CS-ACO1*), AF033582 (*CS-ACO2*) and AF033583 (*CS-ACO3*).

sex-determining genes seem to act by advancing, or delaying the female sex phase, or by changing the fate of specific buds. The inheritance of sex expression in cucumber has been worked out (reviewed by Perl-Treves, 1999), and three major genes, *M*, *F*, and *A*, account for most phenotypes (after the newer gene nomenclature for cucurbits; (Robinson *et al.*, 1976). *F* controls femaleness; this partially dominant gene causes the female phase to start earlier. Gynoecious lines (*FFM–*) bear exclusively female flowers, and are used for their high yield and as parental lines in hybrid seed production, while *ffMM* is the ordinary monoecious genotype, displaying a long male phase. The *M* locus affects the presence of stamens in pistillate flowers: *ffmm* plants are andromonoecious, with a male phase followed by bisexual flowers, while *FFmm* genotypes are hermaphrodite, bearing mostly bisexual flowers. The recessive *a* gene intensifies maleness, interacting epistatically with the *F* locus: *ffaa* genotypes are androecious, i.e. bear exclusively staminate flowers and never attain the female phase.

Of particular interest is the relationship between cucumber sex determination and plant hormones, mainly ethylene and gibberellin. Gibberellin treatments induce male flowers on female plants (Galun, 1959a), probably by causing the abortion of female buds and the formation of adventitious male buds (Fuchs *et al.*, 1997). Auxins promote female flower formation (Galun, 1959b), but the strongest feminizing effect is exerted by ethylene (Rudich *et al.*, 1969). Rudich *et al.* (1976) attempted to establish the role of endogenous ethylene in sex expression and reported two-fold rates of ethylene evolution in a gynoecious versus a monoecious line, whereas in an androecious line ethylene was lowest. Trebitsh *et al.* (1987) studied the potential of different genotypes to evolve ethylene by supplying ACC to excised apices; the results suggested more ethylene-forming activity, as well as higher ACC endogenous content in gynoecious plants. In a recent study by Trebitsh *et al.* (1997), gynoecious cucumbers appeared to have an additional copy of ACC synthase, as compared to monoecious genotypes, and this gene was mapped to the *F* locus. The tight linkage suggested that such clone may be the *F* gene itself. *CS-ACS1* transcripts were auxin-inducible in cucumber shoot apex and leaves, but their levels were similar in monoecious and gynoecious genotypes, and it remains to be seen how, and where, does the extra copy exert its presumed effect on sex expression.

ACC oxidase ('ethylene-forming enzyme') catalyses the second, final step in ethylene formation, following ACC production by ACC synthase, and its expression is finely regulated in plants (Barry *et al.*, 1996; Kim *et al.*, 1997). We set to study the ACC oxidase (ACO) gene family in cucumber plants, trying to correlate the expression of ACO genes to the sex patterns found in the various sex genotypes.

Materials and methods

Plant material

Cucumber lines for this study were provided by E. Galun, Weizmann Institute of Science, Rehovot, and S. Niego, Zeraim Gedera Seed Company, Israel. Four genotypes displaying well characterized sex-expression patterns were chosen: Elem Female, a gynoecious breeding line, genotype *FFMMAA*, that produces only female flowers starting from the first nodes; Erez, an androecious line of similar genetic background, genotype *ffMMaa*, producing exclusively male flowers; Shimshon, a monoecious cultivar, genotype *ffMMAA*, displaying typical sex phases; Michigan State University line 319H, a hermaphrodite line having bisexual flowers (occasionally accompanied by male flowers) from the bottom-most nodes, genotype *FFmmAA*. Plants were grown in 10 litre pots in a glasshouse during the winter (18 °C night, 30 °C day) or in a net-house in spring and summer, under natural illumination. For ethrel induction experiments, plants in the third leaf stage were given a single spray of 1.2 mM ethrel (2-chloroethyl phosphonic acid; AGAN Chemical Manufacturers, Israel), a concentration used by breeders for sex reversal, supplemented with 0.1% Tween 20 as wetting agent.

Isolation and sequence analysis of ACC oxidase-encoding cDNAs

cDNA libraries from cucumber male and female floral buds at a young (1 mm size) developmental stage (Perl-Treves *et al.*, 1998a) were plated and screened at moderate stringency (6 × SSC, 55 °C) with a tomato ACC oxidase probe (Hamilton *et al.*, 1991) according to standard procedures (Ausubel *et al.*, 1987). Three clones, containing different sequences, were selected for further study, subcloned and sequenced on both strands (Perl-Treves *et al.*, 1998b). The cDNAs were named *CS-ACO1*, *CS-ACO2* and *CS-ACO3*. *CS-ACO1* and *CS-ACO3* encoded full-length reading frames, while *ACO2* was truncated at the 5′ end. A longer

CS-ACO2 clone was then isolated, and the respective GenBank accession has been updated. Sequence comparisons and alignment were carried out using the GCG computer programs GAP and PILEUP of the University of Wisconsin, Genetics Computer Group Package (Devereux, 1989). Cluster analysis with other *ACO* sequences was performed using the PROTDIST and NEIGHBOR programs of the PHYLIP software package (Felsenstein, 1993).

Northern and Southern blot analysis

Total RNA for northern analysis was extracted using the Tri-reagent Kit (Molecular Research Center, Ohio) according to the manufacturer's instructions. RNA was separated on agarose-formaldehyde gels (Ausubel *et al.*, 1987) and blotted onto Nylon membranes (Magna, MSI). Hybridization was in 0.33 M sodium phosphate pH 7, 10 mM EDTA, 5% SDS, 170 μg/ml sheared salmon DNA and 10% dextran sulfate, at 60 °C overnight, followed by washes at 60 °C in 2 × SSPE, 0.5% SDS for 30 min, and 0.5 × SSPE, 0.5% SDS for 15 min. To visualize total RNA, membranes were stained with methylene blue (Sambrook *et al.*, 1989). To control the specificity of northern hybridization, we prepared unlabelled *in vitro* transcription reactions using the ACO1,2,3 linearized plasmids as templates for T7 and T3 RNA polymerases (Promega), according to the manufacturer's instructions. Of the reaction products 1 μg aliquots were run on agarose gels and stained with ethidium bromide in order to estimate the integrity and concentration of the transcripts. About 10 ng of each of the three 'sense' (mRNA-like) transcripts were loaded onto agarose-formaldehyde gels, blotted and hybridized to ACO DNA fragments under the same conditions used for the plant RNA blots. Genomic DNA for Southern analysis was extracted according to Baudracco-Arnas (1995). DNA (3 μg/lane) was restriction-digested, run through a 1% agarose gel, and alkali-blotted onto Genescreen Plus membranes (DuPont). Hybridization was at 68 °C in 5 × SSPE, 5× Denhardt's solution, 1% SDS, 100 μg/ml salmon sperm DNA (Ausubel *et al.*, 1987). Blots were washed at increasing stringency up to 65 °C in 0.1 × SSC, 0.1% SDS. Probes for northern and Southern analysis were prepared by ^{32}P radiolabelling of PCR-amplified plasmid inserts, using the Klenow enzyme (Random Hexamer method, Boehringer Mannheim protocol).

Linkage analysis

For linkage analysis, an F_2 population segregating for the *F* gene was produced by crossing Elem Female (gynoecious) with Shimshon (monoecious). A single monoecious F_1 plant was self-pollinated and F_2 seeds were sown in the greenhouse. Over 100 F_2 individuals were scored weekly for their sex pattern along the main stem and lateral branches. Genotype assignment was as follows: individuals that were fully gynoecious, without any male flower on either the main shoot or the side-branches (starting from node number 1–3, since the first 1–2 nodes may be barren), were scored as *FF*. Monoecious individuals, scored as *ff*, had a long male phase (first female flower on the main shoot appeared at node number 12.7 ± 3.8), followed by a mixed phase, and no continuous-female phase was observed; the proportion of male flowers in the first 20 nodes was 90% ± 3%. Plants scored as *Ff* heterozygotes displayed a monoecious sex type, i.e. they did have a variable proportion of male flowers (35% ± 20%). They were easy to distinguish also from the *ff* class, since the first female flower formed at a lower node number (2.9 ± 2.0), and a continuous-female phase was attained. Leaf samples for DNA extraction were harvested, and Southern analysis was performed to detect RFLP with the *CS-ACO2* probe.

In situ *mRNA hybridization*

In situ mRNA hybridization was carried out according to Jackson (1997). Flower buds of different developmental stages were collected from greenhouse-grown plants, fixed in paraformaldehyde and embedded in Wax Paramat extra-pastillated (BDH Gurr). Buds were microtome-dissected into 10 μm slices and tissue sections were attached to Super-Frost Plus slides (Menzel-Glaser). Hybridization probes were prepared by *in vitro* transcription of linearized Bluescript plasmids harbouring the *ACO* cDNAs, using T7 and T3 RNA polymerase (Promega) according to the manufacturer's protocol, in the presence of digoxigenin (DIG)-UTP (Boehringer Mannheim). Probes were alkali-hydrolysed to ca. 150 bp, and transcript concentration was estimated by dot-blotting serial dilutions of the probe alongside a DIG-labelled control RNA (Boehringer Mannheim) onto Hybond N+ membrane (Amersham), followed by a DIG detection reaction. Slides with tissue sections were pre-treated with pronase and cross-linked with acetic anhydride (Merck). Hybridization was carried out overnight at 50 °C in 50% deionized formamide, 10% dextrane

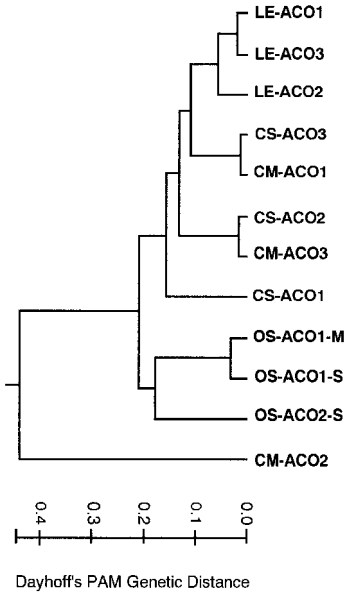

Figure 1. Cluster analysis of the *ACO* gene family from different plants. The following sequences were analysed using the PROTDIST and NEIGHBOR programs: *CS-ACO1*, GenBank accession number AF033581; *CS-ACO2*, AF033582; *CS-ACO3*, AF033583 (Perl-Treves *et al.*, 1998b); *CM-ACO1*, Q04644 (Balague *et al.*, 1993); *CM-ACO2*, X95552; *CM-ACO3*, X95553 (Lasserre *et al.*, 1996); *LE-ACO1*, P05116 (Köck *et al.*, 1991); *LE-ACO2*, P07920 (Holdsworth *et al.*, 1987); *LE-ACO3*, P24157 (Spanu *et al.*, 1991); *OS-ACO1*-S, AF049888; *OS-ACO2*, 49889; *OS-ACO1*-M, X85747.

sulfate, 1× Denhardt's solution, 1 mg/ml tRNA, 0.3 M NaCl, 10 mM Tris-HCl pH 6.8, 5 mM EDTA, 10 mM sodium phosphate buffer and ca. 10 pg/μl RNA probe. Post-hybridization washes included 50% formamide, 2× SSPE washes for 1.5 h each at 50 °C, and a 0.01 mg/ml RNAseA treatment at 37 °C for 30 min. Probe detection was carried out using anti-DIG antibody::alkaline phosphatase conjugate (Boehringer Mannheim). Negative controls were hybridized with 'sense probes', i.e. the same plasmid was transcribed to produce mRNA-like strands. Sections were also stained histologically with Safranin-Fast Green (Sass, 1951).

Results

Identification of cucumber ACC oxidase cDNAs

Three cDNA sequences encoding different ACC oxidases, *CS-ACO1*, *CS-ACO2* and *CS-ACO3*, were isolated from a cDNA library constructed in our laboratory from early-stage female flower buds (Perl-Treves *et al.*, 1998b). The amino acid sequences encoded by the three cDNAs were aligned, and cluster analysis of the cucumber sequences with respect to the homologous sequences of three other *ACO* gene families, those of melon, tomato and rice, was performed (Figure 1). The proportion of identical amino acids among the three cucumber clones is between 73% and 83%. *CS-ACO2* is 97% identical in its amino acid sequence to the melon *CM-ACO3*, while *CM-ACO1* is the melon orthologue of cucumber's *CS-ACO3* (98% identity). None of the melon sequences was closely related to cucumber *CS-ACO1*, nor could we find such a sequence among the numerous *ACO*s from all other species in the database. Melon's *CM-ACO2* is even more diverged, with less than 50% amino acid identity with any other published *ACO*. If we assume that melon and cucumber share a similar genomic complement, there probably exists at least one additional gene family member in both cucumber and melon. A BLAST search for ACO-related sequences in the database reveals other oxidases that are significantly related to ACO. The closest ones included flavone synthases from various plant species (e.g. GenBank accession number U72631 from *Arabidopsis*), flavonone 3-hydroxylases (e.g. AF022142 from *Petunia*), *E8*-related genes from tomato (AF004914) and gibberellin-20 oxidase (e.g. U61385 from *Cucurbita*). The amino acid identity between these oxidases and cucumber *ACO1*,*2*,*3* ranged between 30% and 35%, showing that none of the cucumber clones is likely to represent another oxidase: amino acid identity among the *ACO* sequences from different species shown in Figure 1 was at least 63%. Melon's *ACO2* may represent an exception, with only 45–50% identity with the other *ACO*s, and its identification as an ACC oxidase has been discussed by Lasserre *et al.* (1996); its homology with non-ACO oxidases was nevertheless lower, around 30% identity. The ACO sequences from the other two genera, *Oryza* and *Lycopersicon*, were more distant from the *Cucumis* counterparts. The fact that the three tomato sequences clustered away from the rest of the genes, as did the three rice sequences, could indicate late divergence of the respective genes, after plant-family diversification. On the other hand, given the higher diversity among *Cucumis ACO*s, we may anticipate that the other genera would also have such diverged genemembers, but these have not yet been cloned.

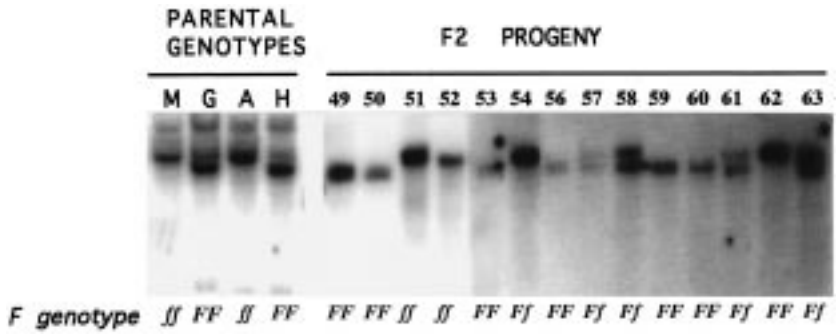

Figure 2. Linkage between *CS-ACO2* and the female locus, *F*. Genomic DNA from four cucumber genotypes (M, monoecious; G, gynoecious; A, androecious; H, hermaphrodite; genotypes listed in Materials and methods) was digested with *Eco*RV restriction endonuclease, along with DNA from F_2 individuals derived from a cross between the gynoecious and the monoecious genotypes. The genotype of the plants with respect to the *F* locus is indicated. Southern hybridization was performed at 68 °C, 5× SSPE, with the *CS-ACO2* cDNA as probe.

Linkage of CS-ACO2 *to the* F *locus*

Southern blots with DNA of four sex genotypes, digested with several restriction enzymes, were prepared and hybridized at high stringency with *CS-ACO* probes. The four contrasting genotypes included a gynoecious, a hermaphrodite, a monoecious and an androecious line. They can be classified according to their *F* gene complement: the first two are *FF* homozygous, the latter two *ff* homozygous. Interestingly, an *Eco*RV RFLP detected with the *ACO2* probe divided them according to the *F* genotype (Figure 2), implicating possible linkage between *CS-ACO2* and the femaleness locus. We therefore prepared *Eco*RV blots of 58 F_2 individuals from a population that segregates for *F*, derived from a cross between a gynoecious and a monoecious line. Most individuals could be classified unambiguously (as detailed in Materials and methods), and we observed clear linkage between *CS-ACO2* and *F* (Figure 2). Seven out of 58 plants were scored as recombinant, corresponding to a map distance of 8.7 cM (LOD score 13.2) between the two genes. Because of the low levels of polymorphism among cucumber genotypes, we did not find any useful polymorphism between the parental lines with the *ACO1* and *ACO3* probes.

Expression analysis of ACO *in developing flowers by* in situ *hybridization*

Based on preliminary northern blot data, *CS-ACO1* exhibited complex transcript patterns and was set aside for future studies, while the two clones, *CS-ACO2* and *CS-ACO3*, were selected for the present analysis. We wondered whether the respective genes are expressed in a tissue-specific pattern in developing male and female flowers of cucumbers, where the ultimate response to sex determination programmes takes place. Male and female floral buds representing a sequence of developmental stages were fixed, dissected and hybridized to *ACO2* and *ACO3* probes. In young, 1.5 mm long male buds (Figure 3A–C), *CS-ACO3* was strongly expressed in well-defined zones, adjacent to developing vascular bundles, where differentiating tracheary elements are seen. A similar pattern was observed with *ACO3* in young, 2 mm long female buds (not shown). At such an early stage, expression patterns of *CS-ACO2* were unclear. In male buds at an intermediate stage of development (5 mm), specific *CS-ACO3* expression was detected in the petal and inner tube tissue, while in mature flowers (one day before anthesis), *CS-ACO3* expression was very weak, and present mainly in the petals and around the nectary (not shown). At this stage, *CS-ACO2* transcripts rose and accumulated in the nectary and pollen grains (Figure 3D–E).

In developing female flowers at the intermediate stage (4 mm buds, Figure 3F, G), stronger and localized *CS-ACO3* expression was detected in the transmitting tissue of the stigmas (but not at the stigmatic surface), in the pistil and nectary regions and, interestingly, in the staminoids, i.e. the inhibited stamen primordia (Figure 3F), where a role for ACO activity in locally inhibiting their further growth may be suggested; note that also in male flowers we found localized expression in the nectary, which develops from the vestigial carpel. *CS-ACO3* was also expressed in the petals, but not (or less so) in sepals. *CS-ACO2* exhibited weak expression at early female-bud stages, but increased in mature female flowers dissected one day before anthesis. At this stage, *CS-ACO3* expres-

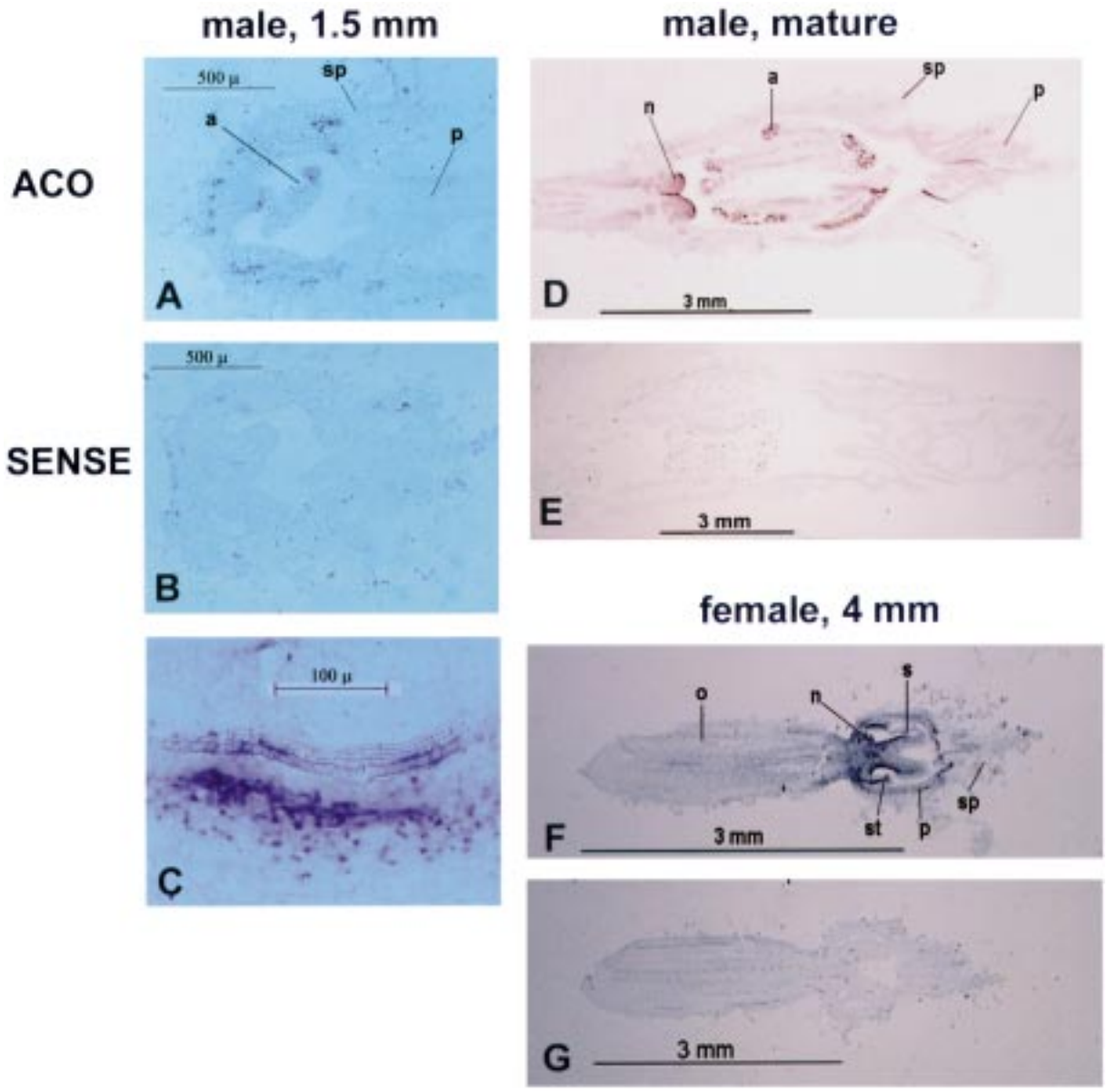

Figure 3. Localization of *CS-ACO2* and *CS-ACO3* transcripts in developing male and female flowers of cucumber. Floral buds were paraffin-embedded, cut into longitudinal sections (10 μm thick), and hybridized with digoxigenin-labelled RNA probes. s, stigma; o, ovary; p, petals; sp, sepals; n, nectary. A. Young, 1.5 mm long male buds hybridized with the *CS-ACO3* antisense probe. B. Same bud, hybridized as a negative control with a *CS-ACO3* sense probe. C. Higher magnification of similar bud, showing localization of *CS-ACO3* transcript along developing vascular tissue. D. Mature male flower (one day before anthesis), hybridized with the *CS-ACO2* antisense probe. E. Same bud, hybridized as a negative control with the sense probe. F. Female bud 4 mm long; hybridized with the *CS-ACO3* antisense probe. E. Same bud, hybridized as a negative control with a *CS-ACO3* sense probe.

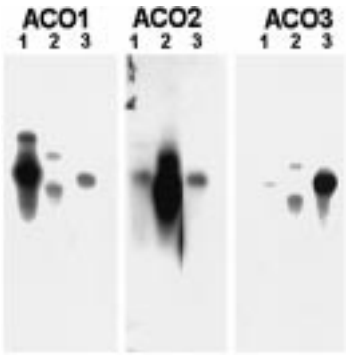

Figure 4. Northern blot hybridization of *in vitro* transcripts to assess *ACO* probe specificity. The *CS-ACO1, -2* and *-3* plasmids were linearized and transcribed *in vitro* to produce unlabelled mRNA-like transcripts. Equal amounts of each transcript (1, *ACO1*; 2, *ACO2*; 3, *ACO3*) were loaded in each lane and triplicate formaldehyde-agarose gels were prepared, blotted and hybridized with the ^{32}P-labelled cDNA probe indicated above each blot. Hybridization conditions were similar to those applied for the northern blots in Figures 5–7.

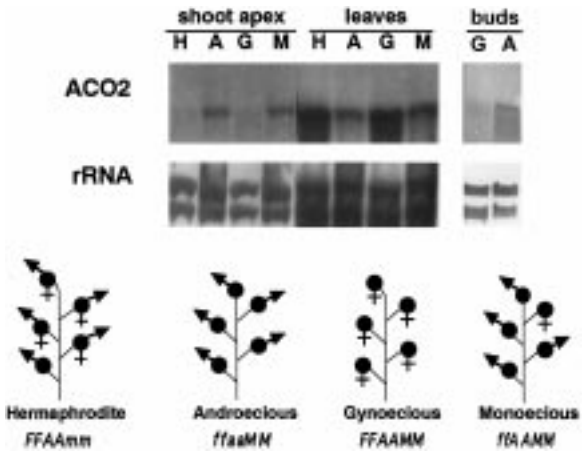

Figure 5. Expression of *CS-ACO2* in leaves, apices and buds of four sex genotypes. Total RNA (10 μg/lane) was extracted from the shoot apex and from the youngest unfolded leaf of cucumber plants from four sex genotypes at the third leaf stage. Young (1 mm, morphologically bisexual) floral buds from the gynoecious and androecious genotypes were analysed as well. Northern blot hybridization was performed with the *CS-ACO2* probe. Methylene blue staining of the blot is shown as a loading control. The four genotypes used for this experiment are depicted below, with a schematic representation of the sex patterns along their shoots.

sion had become weaker, while stronger *CS-ACO2* expression was found in the ovary and the ovules. Localized expression was also apparent in the staminoids (arrested stamens) and in a band of adjacent petal tissue (not shown). Negative control slides reacted with 'sense strand' probes stained very lightly, confirming the specificity of the signals observed with antisense probes. In conclusion, the two genes exhibited differential patterns of expression, both with respect to the tissue and to the stage in which they accumulated.

Differential expression among sex genotypes

The endogenous ethylene level that is believed to underlie sex determination may be modulated, either in the bud or in its vicinity, to affect bud differentiation. A preliminary characterization by northern analysis revealed that all three *ACO* mRNAs are expressed in leaves and shoot apices of cucumber plants. To test the specificity of the hybridization signals obtained with different *ACO* gene probes on northern blots, we performed a control experiment. The *ACO1, 2, 3* plasmids were transcribed *in vitro*, and, triplicate northern blots were prepared with equal amounts of each of the three mRNA-like transcripts. Each blot was hybridized with one of the *ACO* probes under the same stringent conditions employed for northern analysis. Figure 4 shows that the signal obtained by specific hybridization of a cDNA to its own transcript is stronger (about 50-fold) than the weak cross-hybridization signals with the two other gene probes. Since the strength of hybridization signals obtained in leaf and apex samples with each of the three probes is within the same order of magnitude, we conclude that the non-specific component in the signals that we observed on northern blots (Figures 5–7) is very small and the signals can be taken as gene-specific.

In order to look for a possible correlation between sex expression and *ACO* gene expression we decided to follow the levels of two transcripts, *CS-ACO2* and *CS-ACO3*, in the shoot apex, where new floral buds sequentially form, and where the fate of each young bud must be decided in response to internal and external cues, and in the leaf adjacent to the apex. Four cucumber genotypes displaying contrasting sex patterns (gynoecious, monoecious, hermaphrodite and androecious) were compared with respect to *CS-ACO2* transcript levels in their shoot apices and leaves (Figure 5). Such levels differed markedly at the third-leaf stage among the genotypes and between the two organs tested. In the first unfolded leaf, levels were higher in the gynoecious and hermaphrodite plants. Such an observation correlates positively with the *F*-locus haplotype of the lines, in line with the hypothesis that femaleness is associated with increased ethylene evolution. Transcript levels in shoot apices displayed, however, an inverse correlation with femaleness: the two *FF* genotypes (which had a higher *CS-ACO2* sig-

nal in the leaf) had a lower signal in the apex, while the two *ff* genotypes had higher transcript levels in the apex. The experiment was repeated three more times with different plant batches, and similar results were obtained. In order to test whether the difference between whole apices also exists between young, morphologically bisexual flower buds (1 mm long) that reside in the apex and respond to sex determination signals, we collected male and female buds from the androecious and the gynoecious genotypes, respectively. Upon hybridization with the *CS-ACO2* probe (Figure 5, 'buds'), we observed a stronger signal in the genetically male buds, similar to the difference observed between whole apices. A similar expression pattern in leaves and shoot apices was also observed with *CS-ACO3* (not shown).

We then asked whether expression at different stages of monoecious plant development varies according to the sex-phase change that such a plant undergoes. The cultivar we used, Shimshon, develops under the present set of environmental conditions clusters of male flowers at the first 12–15 nodes of the main shoot, then single female flowers, interspersed with male-flower nodes. Groups of monoecious cucumber plants were grown, and their apices, and the first-unfolding leaf below the apex, were harvested at varying plant-age stages. Figure 4 shows that the level of *ACO2* mRNA is high in apices of plants at the 4th, 10th and 13th leaf stage. In shoot tips taken from plants that were unfolding their 16th leaf, after visible transition to the mixed-female phase has occurred, the level of *CS-ACO2* transcript decreased substantially. Again, the situation in leaf samples contrasted with that in the apex: transcript abundance was constant, and lower, in the first three stages, dramatically increasing in the 16th-node leaf to a level higher than in the apex. *CS-ACO3* displayed a similar pattern of expression (not shown). In summary, a positive correlation between *ACO* transcript levels and femaleness was observed in the young leaves, and a negative correlation in the shoot tips.

Response of CS-ACO2 *and* CS-ACO3 *transcripts to ethrel treatment*

The lower levels of *ACO2* and *ACO3* mRNAs in the shoot apex in the monoecious female phase, and in enhanced-femaleness genotypes, may reflect a localized feedback inhibition phenomenon, where higher endogenous ethylene levels in the apex might inhibit its own synthesis. Young leaves, on the other hand,

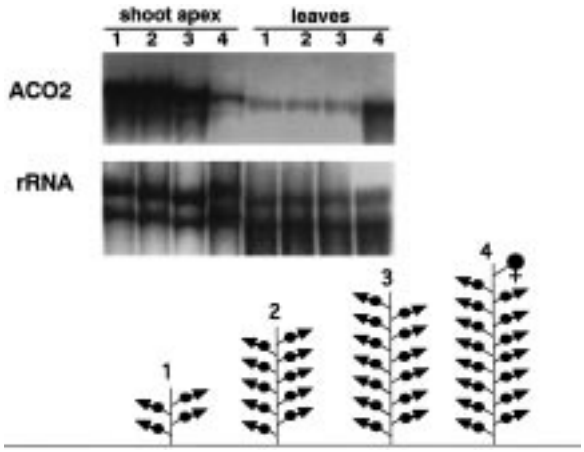

Figure 6. Variation in *CS-ACO2* transcript level along the shoot of monoecious cucumber. Groups of monoecious cucumber plants (cv. Shimshon) were grown, and their shoot tips and youngest unfolded leaf were harvested at varying developmental stages (at the 4th, 10th, 13th and 16th leaf stages). The respective sex patterns along the main stem at the different stages are schematically illustrated. Total RNA (10 μg/lane) was extracted and northern blot hybridization was performed with the *CS-ACO2* probe. Methylene blue staining of the blot is shown as a loading control.

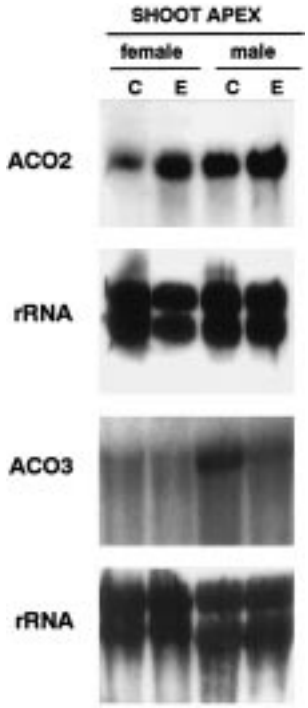

Figure 7. Response of *CS-ACO2* and *CS-ACO3* to ethrel treatment. Young (third-leaf stage) gynoecious ('female') and androecious ('male') plants were treated with ethrel, and after two days the shoot apices were harvested for total RNA extraction. Northern blot hybridization was performed with the *CS-ACO2* and *CS-ACO3* probes comparing treated (E) and untreated control (C) samples. Methylene blue staining of the blot is shown as a loading control.

would not display such an inhibition. In order to test this hypothesis, we treated young androecious and gynoecious plants at the third-leaf stage with ethrel, an ethylene-releasing compound, and harvested their apices and unfolding leaves two days after the treatment. Figure 7 displays the results of the northern hybridization of apex samples with the two probes, *CS-ACO2* and *CS-ACO3*. Comparison of the untreated controls of the two genotypes confirms the results shown in Figure 5: levels of both gene transcripts are lower in the female apex. As for the response to exogenous ethrel, the two genes displayed strikingly different patterns. *CS-ACO2* apparently exhibits a positive response to ethylene, and its levels increased in both genotypes following the treatment. The *CS-ACO3* transcript, on the other hand, exhibited a differential response between the two genotypes: in the male apex, transcript levels decreased upon ethrel treatment, while in the female they remained unchanged. In the young leaf samples of both genotypes (not shown), transcript levels of both genes were elevated in response to ethrel. The data suggest that feedback inhibition of *ACO* genes by ethylene may indeed exist, but only in the shoot apex and only in the case of *CS-ACO3*, while positive feedback regulation seems to act in leaves on both genes, and, in the apex, on *ACO2*: different sex genotypes, plant organs and *ACO* gene-family members respond differentially to exogenous ethrel.

Discussion

The cucumber ACO *gene family*

We have characterized a family of three ACC oxidase cDNAs, isolated from a cucumber female floral bud library. Hybridization of the three probes at high-stringency conditions yielded different genomic patterns. Sequence comparison with the homologous family in melon (Balague *et al.*, 1993; Lasserre *et al.*, 1996) suggests that the *ACO* gene family in both species may include additional gene members.

DNA diversity among cucumber genotypes is notoriously low (Staub *et al.*, 1996), and RFLPs are hard to detect. An *Eco*RV polymorphism detected with the *CS-ACO2* probe intrigued us, because it correlated with the sex genotype among the four cucumber lines examined. F_2 segregation analysis was carried out, and *CS-ACO2* was indeed found to be linked to the *F* locus. Previous work has shown closer, 100% linkage of an ACC synthase gene with *F* (Trebitsh *et al.*, 1997); *CS-ACO2* appears more distant (8.7 cM). We plan to perform more refined mapping of *CS-ACO2*, *CS-ACS1* and *F*, to test the possibility of an 'ethylene-related cluster', or 'sex cluster', in the *Cucumis* genome.

Differential expression of ACO *transcripts in floral organs*

The expression of two family members, *CS-ACO2* and *CS-ACO3*, was studied at the transcript level in more detail. It is clear that the two have distinct expression domains in the flower, at different stages of development (Figure 3). The relative level of expression also changes, with *CS-ACO3* transcripts decreasing towards anthesis, while *CS-ACO2* level seems weaker at young stages, and increases later. Interestingly, strong *CS-ACO3* expression along differentiating vascular elements was observed in very young buds. Roles for ethylene in the control of differentiation and/or growth of tissues in the pistil, ovule and petal, as well as the nectary and pollen, at specific stages of bud development, may be postulated from the *in situ* hybridization patterns. These putative roles must be unrelated to flower senescence or stress phenomena. High ACC oxidase activity, and transcript levels, were associated with actively dividing tissues in pea and sunflower seedlings, respectively (Taylor *et al.*, 1988; Liu *et al.*, 1997). Organ-specific *ACO* expression patterns were demonstrated in developing tomato flowers (Barry *et al.*, 1996). In *Petunia*, two *ACO* genes were specifically expressed in developing pistil tissue (Tang *et al.*, 1993). One of them was strongly expressed in the secretory zones of the stigma and nectary towards anthesis, while a third gene was expressed in all floral organs in response to ethylene, and was apparently related to the senescence program.

It thus appears that several different *ACO* isozymes are expressed in small, distinct floral regions. Multiple genes may be required if a rather complex pattern of spatial, temporal and inductive expression has to be achieved, and ethylene synthesis genes are known to be regulated by a multitude of developmental and environmental signals. Moreover, it has been argued that ethylene production must be controlled on a strictly localized basis (Fluhr and Mattoo, 1996). Because of its volatility, localized production at its site of action is required, especially in the last step, catalysed by *ACO*. The ACC precursor, on the other hand, may be transported in the plant (Woltering, 1990). Localized ethylene bursts were reported during the

geotropic response of *Arabidopsis* seedlings (Harrison and Pickard, 1984), as well as in orchid flowers, where pollination induces, via an ethylene signal, petal senescence, at the same time inhibiting ovary senescence (Nadeau *et al.*, 1993).

It is tempting to interpret the expression of *CS-ACO3*, and later of *CS-ACO2*, in the arrested stamens of the pistillate flowers (and possibly also in the nectariferous ring derived from the arrested pistil in the male flower) as a sex-related phenomenon: is there a local ethylene signal required to keep the 'wrong sex' organs from further development? Observing the same pattern at an earlier bud stage, where stamen arrest may be decisive for the bud's fate, would substantiate such a hypothesis, but such patterns in very small buds were hard to detect. Such a question may be addressed in the future using transgenic plants in which *ACO* gene transcription will be silenced during floral development.

Relationship between femaleness and ACO *expression*

A number of studies reported a correlation between the degree of femaleness in cucumber and endogenous ethylene production. Gynoecious plants evolved 2–3 times more ethylene than monoecious and androecious genotypes (reviewed by Perl-Treves, 1999). Rudich *et al.* (1976, and references therein) showed that detached female floral buds evolved more ethylene than male flower buds. Moreover, the sex-determining locus, *F*, was found to be linked to an *ACS* gene, but no difference in the respective transcript levels was observed between gynoecious and monoecious genotypes (Trebitsh *et al.*, 1997); another *ACS* gene, isolated by Kamachi *et al.* (1997), had an expression pattern that corresponded to the onset of the female phase in monoecious and gynoecious cultivars. In view of these data, our results are rather surprising: ACC oxidases, encoding the last step of ethylene synthesis, are expressed in leaves, shoot tips and flowers, but expression in the apex, where the sex expression response takes place, seems inversely related to femaleness. *CS-ACO* transcripts decreased in the final sex phase in monoecious cucumber plants (Figure 6) and, in younger plants, were higher in *ff* genotypes than in *FF* ones (Figures 5 and 7). Young leaves, on the other hand, contained higher *CS-ACO2* and *-3* transcript levels in female phase/genotypes. We may hypothesize, therefore, that expression of these genes in the apex (as detected at the northern blot level) is not a factor that limits the sex determination process. Other *ACS* or *ACO* genes, probably modulated in a very localized fashion at a critical stage, may be important in generating the elevated ethylene signal required for female differentiation (along with a lower ethylene signal probably required for male differentiation). Such a differential ethylene signal may be produced somewhere in the apex or the floral bud in spite of, or irrespective of, the reduced *CS-ACO2* and *-3* transcript levels in the surrounding tissue. A different interpretation would be that ethylene production that determines sex expression takes place in the young leaves near the apex, where it is positively correlated with *ACO* transcript level.

The correlations observed in this study led us to suggest that a negative feedback inhibition of *ACO* gene transcription may operate in the apex, while a positive one may operate in the leaf. The elevation of transcript levels in the leaves following ethrel application probably reflects the well-known 'autocatalytic' property of ethylene, that induces its own synthesis in many experimental systems (Tang *et al.*, 1993; Barry *et al.*, 1996; Have and Woltering, 1997). There are, nevertheless, documented cases in which ethylene inhibits its own production, e.g. during banana and *Ficus* fruit maturation (Vendrell and McGlasson, 1971; Zeroni *et al.*, 1976), and possibly also in sunflower seedlings, where inhibitors of ethylene action increased the expression of *ACO* (Liu *et al.*, 1997). In the present study, both a positive and a negative response to exogenous ethrel were apparent, with the two genes, *CS-ACO2* and *CS-ACO3*, responding differentially in the shoot apex (Figure 7). *CS-ACO2* transcript levels increased after the treatment, while *ACO3* levels decreased in the male apex (where its level was originally higher), and remained unaffected in the female apex. It is therefore possible that the inverse correlation between femaleness and *ACO* expression in untreated apices results from such a feedback inhibition phenomenon: when endogenous ethylene is high (in the female apex) the *CS-ACO* gene is repressed, whereas in male tissue, where ethylene is low, *ACO* genes are not repressed. However, only the response of the *ACO3* transcript may support such a model.

In conclusion, the relationship between *ACO* transcript levels and sex expression patterns in cucumber is not a simple one, and further research will be required to elucidate the role played by ethylene synthesis and ethylene perception in this developmental process. The patterns of expression of individual

ACO genes in cucumber emerging from this study are highly specialized, and appear to be regulated both by tissue-specific cues and by endogenous and exogenous ethylene. The complexity of ethylene response has been demonstrated in a recent study on ethylene-responsive promoters, where the same DNA element imparted a different response in leaves versus reproductive organs (Deikman *et al.*, 1998). Complex regulatory interactions must be there to provide the fine coordination between the many roles, both promotive and inhibitory, that are probably played by ethylene during cucumber floral development.

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